Regulation of Hepatic Phase II Metabolism in Pregnant Mice[S]

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ABSTRACT

Phase II enzymes, including Ugts, Sults, and Gsts, are critical for the disposition and detoxification of endo- and xenobiotics. In this study, the mRNA and protein expression of major phase II enzymes, as well as key regulatory transcription factors, were quantified in livers of time-matched pregnant and virgin control C57BL/6 mice on gestation days (GD) 7, 11, 14, 17, and post-natal days (PND) 1, 15, and 30. Compared with virgin controls, the mRNA expression of Ugt1a1, 1a6, 1a9, 2a3, 2b1, 2b34, and 2b35 decreased 40 to 80% in pregnant dams. Protein expression of Ugt1a6 also decreased and corresponded with reduced in vitro glucuronidation of bisphenol A in S9 fractions from livers of pregnant mice. Similar to Ugts levels, Gsta1 and a4 mRNAs were reduced in pregnant dams in mid to late gestation; however no change in protein expression was observed. Conversely, Sult1a1, 2a1/2, and 3a1 mRNAs increased 100 to 500% at various time points in pregnant and lactating mice and corresponded with enhanced in vitro sulfation of acetaminophen in liver S9 fractions. Coinciding with maximal decreases in Ugts as well as increases in Sults, the expression of transcription factors CAR, PPARα, and PXR and their target genes were downregulated, whereas ERα mRNA was upregulated. Collectively, these data demonstrate altered regulation of hepatic phase II metabolism in mice during pregnancy and suggest that CAR, PPARα, PXR, and ERα signaling pathways may be candidate signaling pathways responsible for these changes.

Introduction

Phase II enzymes, including UDP-glucuronosyltransferases (Ugts), sulfotransferases (Sults), and glutathione S-transferases (Gsts), detoxify various endo- and xenobiotics to more hydrophilic compounds that are excreted in bile and urine. Phase II enzymes are mainly expressed in the liver, with some isoforms also expressed in the gastrointestinal tract, kidneys, and adipose tissue (Bock, 2010; Alnouti and Klaassen, 2011). The regulation of phase II enzyme expression is complex and involves multiple signaling mechanisms. It is becoming increasingly evident that sex-specific hormones and diurnal patterns of hormone secretion are important in regulating phase II enzymes. For example, livers of female mice predominantly express Ugt1a1 and 1a5 mRNA, and livers of male mice predominantly express Ugt2b1 mRNA (Buckley and Klaassen, 2009b). Sex divergent expression of specific Ugt isoforms is largely due to different patterns of growth hormone secretion between male and female mice. Likewise, Sult1a1, 1d1, and 2a1/2 mRNAs exhibit female-predominant expression in liver because of the suppressive effects of androgens and growth hormone secretion in male mice (Alnouti and Klaassen, 2011). Therefore, up- or downregulation of phase II enzymes by fluctuations in sex hormones may be important during periods such as pregnancy (Luquita et al., 2001; Sweeney et al., 2006; Papacleovoulou et al., 2011).

During pregnancy, there are a number of maternal physiologic alterations including elevated levels of female sex steroid hormones, such as estradiol and progesterone, as well as changes in the metabolism of lipids and cholesterol (Belo et al., 2004; Freemark, 2006; Papacleovoulou et al., 2011). Fluctuations in hormone levels and lipid metabolism during pregnancy may alter the activity of hepatic nuclear receptors that control the expression of phase II enzymes. In general, phase II enzymes are cross regulated by a group of transcription factors including the aryl hydrocarbon receptor

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; APAP, acetaminophen; bDNA, branched DNA; BPA, bisphenol A; CAR, constitutive androstane receptor; ERα, estrogen receptor alpha; GD, gestation days; Gsts, glutathione S-transferases; HPLC, high-performance liquid chromatography; HNF4α, hepatocyte nuclear factor 4 alpha; Nqo1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor e2-related factor 2; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PND, postnatal days; Pgc-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor; qPCR, quantitative PCR; Sults, sulfotransferases; UDPGA, uridine 5′-diphosphoglucuronic acid; Ugts, UDP-glucuronosyltransferases; V, virgin controls.

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(AhR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor alpha (PPARα), pregnane X receptor (PXR), and the estrogen receptor alpha (ERα) (Bock, 2010; Tolson and Wang, 2010; Alnouti and Klaassen, 2011, 2012). In addition, the hepatic transcription factors, nuclear factor e2-related factor 2 (Nrf2) and hepatocyte nuclear factors (HNF), are also involved in coordinately regulating phase II enzymes (Aleksunes and Manautou, 2007).

Previous studies have demonstrated decreased hepatic nuclear receptor expression in pregnant mice and rats that corresponds with downregulation of several metabolic enzymes and hepatobiliary transporters (Jeong, 2010; Milona et al., 2010; Papacleovoulou et al., 2011). However, a systematic mRNA profiling of phase II enzyme isoforms and regulatory transcription factors at multiple time points during pregnancy and lactation in mice is lacking. Therefore, in the present study, the mRNA and protein expression of conjugation enzymes, as well as potential regulatory transcription factors, were quantified in livers of time-matched pregnant and virgin control C57BL/6 mice throughout pregnancy and lactation. By using bisphenol A (BPA) and acetaminophen (APAP) as marker substrates, phase II enzyme activities were also assessed in S9 fractions from livers of virgin control and pregnant mice at gestation days 14 and 17. Mice were selected for this study because of their increasing use in reproductive and developmental toxicology. Understanding the transcriptional regulation of phase II enzymes may guide in prescribing drugs during pregnancy to prevent excessive fetal exposure to harmful chemicals and avoid maternal toxicities.

Materials and Methods

APAP, BPA, uridine 5′-diphosphoglucuronic acid (UDPGA, purity >99%) and 3′-phosphoadenosine-5′-phosphosulfate (PAPS, purity >60%) were purchased from Sigma-Aldrich (St. Louis, MO). BPA-glucuronide was obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Authentic APAP standards were kindly provided by McNeil-PPC, Inc. (Fort Washington, PA) in collaboration with Dr. José Manautou at the University of Connecticut. Other chemicals and reagents were obtained from Sigma-Aldrich.

Animals. Adult female and male C57BL/6 mice (027 strain; Charles River Laboratories, Inc., Wilmington, MA) were mated overnight, and males were removed the next morning. This time point represented GD0. Mice were provided food and water ad libitum. On GD 7, 11, 14, and 17 and postnatal days (PND) 1, 15, and 30, serum and liver were collected from pregnant mice and time-matched virgin controls (V). Parturition occurred between GD19 and 21. Pups were housed with dams until weaning on PND21. Livers were harvested and snap frozen in liquid nitrogen. Tissues were stored at −80°C until use. The Institutional Animal Care and Use Committees at Rutgers University and the University of Kansas Medical Center approved these studies.

RNA Isolation and Messenger RNA Quantification. Total RNA from livers of virgin control, pregnant, and lactating mice was isolated using RNABee reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Concentrations of total RNA were quantified by Nanodrop spectrophotometry (Fisher Sci, Pittsburgh, PA) at 260 nm.

Messenger RNA was quantified either by branched DNA (bDNA) signal amplification (Buckley and Klaassen, 2007; Petrick and Klaassen, 2007; Cui et al., 2010; Alnouti and Klaassen, 2011) or quantitative real-time PCR (qPCR) depending on the availability and specificity of primers. The mRNA expression of mouse Ugt1a1, 1a5, 1a9, 2a3, 2b1, 2b5, 2b34 and 2b35, Sult1a1, 1d1, 2a1/2 and 3a1, Gsta1, a4, m2 and p1, transcription factors and their target genes including

AhR, cytochrome P450 1a1 (Cyp1a1), CAR, Cyp2b10, PPARα, Cyp4a14, PXR, and Cyp3a11 was quantified using the bDNA signal amplification assay (Panomics QuantiGene, High Volume bDNA Signal Amplification Kit 1.0; Affymetrix, Santa Clara, CA). Multiple oligonucleotide probe sets (containing capture, label, and blocker probes) specific to mouse mRNA transcripts were designed using ProbeDesigner software (version 1.0; Bayer Corp., Diagnostics Division, Tarrytown, NY).

The hepatocyte nuclear factor 4-alpha (Hnf4α), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1α), ERα, ERβ, and progesterone receptor were quantified by qPCR. Complimentary DNA (cDNA) was generated with the First Strand SuperScript cDNA synthesis kit (Invitrogen, Carlsbad, CA). Specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) for each gene (see Supplemental Table 1) were added to 1μg of cDNA from each sample. Sybr Green (Applied Biosystems, Carlsbad, CA) was used for detection of amplified products. qPCR was performed in a 384-well plate format using the ABI 7900HT PCR system (Applied Biosystems). The threshold cycle (Ct) value was converted to ΔΔCt values by normalizing to β-actin.

Western Blot Analysis. Mouse livers from virgin and pregnant mice at GD14 and 17 were homogenized in buffer containing 10 mM Tris base and 150 mM sucrose, pH 7.5. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Homogenates (50 μg) were separated by electrophoresis on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), then transferred to polyvinylidene fluoride membranes and blocked with 5% nonfat dry milk in 0.5% PBST (PBS buffer with 0.5% Tween 20) for 1 hour. Membranes were incubated with primary antibodies to Ugt1a1 (AB62600), 1a6 (AB97646), 2b10 (AB57685), Sult1a1 (AB57849), Nqo1 (AB2346) (Abcam, Cambridge, MA); Ugt1a9 (H00054600-B01P) (Abvona, Walnut, CA); Sult2a1 (SC-32942), Cyp4a14 (SC-46087) (Santa Cruz Biotechnology, Santa Cruz, CA); Gsta1 (T3370) (Epitomics, Burlingame, CA); Gsta4 (SAB1401164) (Sigma-Aldrich); Cyp2b10 (AB9916), Cyp3a11 (MAB10041) (EMD Millipore, Billerica, MA); and Cyp1a1 (C1+C4, from Dr. Paul E. Thomas) for 1 to 2 hours. After washing with 0.5% PBST, membranes were incubated with species-appropriate secondary antibodies for 1 hour. Membranes were incubated with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA). Relative band intensities were quantified using a FluorChem Imager (Alpha Innotech, San Leandro, CA) and normalized to levels of β-actin protein (Abcam). Of note, primary antibodies against mouse Ugt1a5, 2a3, 2b1, 2b5, 2b34, 2b35, Sult3a1, 1d1, and Gstm1, p1 proteins were not commercially available.

Isolation of Liver S9 Fractions. Mouse livers from virgin and pregnant mice at GD14 and 17 were homogenized in homogenizing buffer (50 mM Tris-HCl, 1.15% KCl, pH 7.4) and centrifuged at 9000 g for 20 minutes. Supernatants (S9 fraction) were stored at −80°C until use in in vitro enzyme assays.

In Vitro BPA Glucuronidation Assay. S9 fractions (15 μg) were incubated with 1 μM BPA and 2 mM UDPGA in 100 μl of 20 mM phosphate buffer, pH 7.0, at 37°C for 30 minutes. At the end of the incubation, samples were heated in boiling water to deactivate enzyme activity and centrifuged at 14,000 g for 10 minutes. Supernatants were collected and subjected to high performance liquid chromatography (HPLC).

In Vitro APAP Glucuronidation and Sulfation Assays. For glucuronidation of APAP, the incubation mixture contained mouse liver S9 fractions (100 μg protein), 10 mM MgCl₂, 1 mM UDPGA, alamethicin (25 μg/mg protein), and 2 mM APAP (dissolved in methanol, final concentration 0.2%) in 200 μl of 50 mM Tris buffer, pH 7.4, as described previously (Wen and Walle, 2006). After 30 to 120 minutes of incubation at 37°C, reactions were terminated by the addition of 200 μl of cold HPLC-grade methanol. For sulfation of APAP, 2 mM APAP was incubated at 37°C for 30 minutes with mouse liver S9 fractions (100 μg) in 200 μl of 50 mM Tris buffer, pH 7.4, containing 0.0625% bovine serum albumin, 8 mM dithiothreitol, and 0.1 mM PAPS (Wen and Walle, 2006). Reactions were terminated by addition of

200 μl of cold HPLC-grade methanol, and samples were centrifuged at 14,000 g for 2 minutes. To quantify APAP-glucuronide and APAP-sulfate formation, supernatants were subjected to HPLC.

HPLC Analysis. BPA and BPA-glucuronide were analyzed using HPLC-UV (System Gold; Beckman Coulter, Inc., Fullerton, CA). Each sample (50 μl) was injected into a ZORBAX SB-Aq column (5μm, 4.6 × 150 mm; Agilent Technologies, Santa Clara, CA). A gradient elution with 80% acetonitrile and 20% 20 mM phosphate buffer, pH 2.5, at a 1 ml/min flow rate was used. BPA and BPA-glucuronide were identified using standards of BPA and BPA-glucuronide dissolved in 20 mM phosphate buffer, pH 7.0. The UV wavelength for BPA and BPA-glucuronide was 200 nm. Quantitation was achieved by comparing the detected peak area to a standard curve.

For APAP glucuronide and sulfate analysis, all samples were analyzed by reverse-phase HPLC system based on previously described methods with modifications (Lickteig et al., 2007; Reisman et al., 2009). The HPLC system (Shimadzu CTO-6A; Shimadzu, Kyoto, Japan) was equipped with a UV detector (Shimadzu SPND-6A) and an Eclipse XDB-C18 column (4.6 mm × 15 cm, 3.5 μm). The UV wavelength for APAP and its conjugates was 254 nm. The flow rate was 1 ml/min.

Mobile phase A contained 8% methanol and 1% acetic acid in water, and mobile phase B contained 50% methanol in water. The initial running condition for HPLC was 100% mobile phase A. The following time and percent of mobile phase B were 5 minutes (0%), 15 minutes (25%), and 20 to 30 minutes (100%). Quantitation was achieved by comparing the detected peak area to an APAP standard curve (Howie et al., 1977).

Data Analysis. Messenger RNA data were normalized to time-matched virgin controls at each time point (set to 1.0). All data were expressed as mean ± S.E. (n = 3–4, for enzyme activity assay n = 5–6). Differences between virgin and pregnant mice of mRNA and protein levels were evaluated by unpaired t tests at each time point and P values ≤0.05 were considered statistically significant using GraphPad Prism software (version 5; GraphPad Software, Inc., San Diego, CA).

Results

Hepatic Ugt Expression and Function during Pregnancy and Lactation. Nine Ugt isoforms (Ugt1a1, 1a5, 1a6, 1a9, 2a3, 2b1, 2b5, 2b34, and 2b35) were selected on the basis
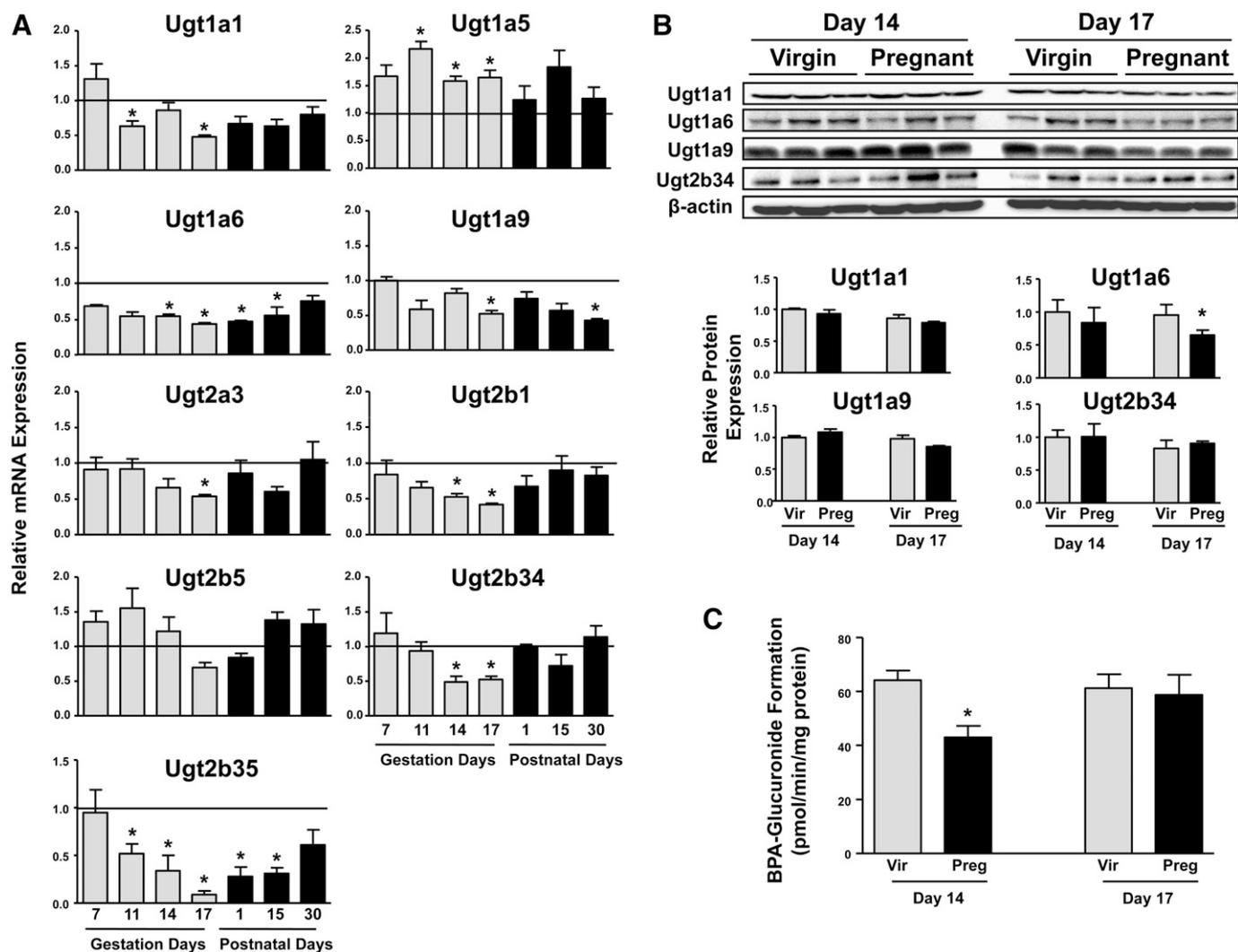

Fig. 1. Expression and activity of hepatic Ugts in pregnant mice. (A) messenger RNA expression of Ugt1a1, 1a5, 1a6, 1a9, 2a3, 2b1, 2b5, 2b34, and 2b35 was quantified by the bDNA assay in time-matched pregnant and virgin control mice at various gestational and postnatal days. Data were normalized to virgin mice (set to 1.0) and presented as mean ± S.E. (n = 3–4). (B) protein expression of Ugt1a1, 1a6, 1a9, and 2b34 was quantified by Western blotting analysis in time-matched pregnant (GD14 and 17) and virgin mice (n = 3). (C) bisphenol A (BPA, 1 μM) was incubated with mouse liver S9 fractions isolated from time-matched pregnant (GD14 and 17) and virgin mice for 30 minutes in the presence of the cofactor UDPGA (n = 5-6). *P < 0.05, statistically significant differences compared with time-matched virgin mice.

of their moderate to high basal expression in female mouse livers (Buckley and Klaassen, 2009b). Ugt mRNA expression was quantified in time-matched pregnant and virgin control C57BL/6 mice on GD7, 11, 14, 17, and PND1, 15, and 30 (Fig. 1A; Table 1). Compared with virgin controls, the mRNA expression of Ugt1a1, 1a6, 1a9, 2a3, 2b1, 2b34, and Ugt2b35 was decreased by 40 to 80% in pregnant dams, with prominent downregulation on GD14 and 17. In pregnant dams, mRNA expression of Ugt1a5 increased by 50 to 100% compared with virgin controls. The mRNA expression of most Ugts isoforms returned to control levels by mid to late lactation, with the exception of Ugt1a9 and 2b35, which remained decreased. Levels of Ugt2b5 mRNA were not changed significantly at any time point.

Because the most remarkable changes in mRNA expression occurred on GD14 and 17, protein expression of select Ugt isoforms was quantified at these time points (Fig. 1B). Protein expression of Ugt1a1 and 1a9 decreased slightly (~20%) on GD17, whereas Ugt1a6 was significantly downregulated by 40%. No change in Ugt2b34 protein was observed at either time point.

BPA is primarily glucuronidated by UGT2B15 in human liver microsomes and by UGT1A9 and 2B7 (Hanioka et al., 2008). The mouse isoforms of UGT2B15 and UGT2B7 are Ugt2b35 and Ugt2b1, respectively. In pregnant mice, Ugt1a9, 2b1, and 2b35 mRNA expression was decreased at GD14 and/

TABLE 1
Summary of phase II enzyme and transcription factor-related mRNA changes in the livers of mice during pregnancy and lactation
Dashes (—) indicate no significant change. Statistically significant changes are designated as ↑↑ or ↓↓.

Days	Pregnancy				Lactation		
	7	11	14	17	1	15	30
Ugts							
Ugt1a1	—	↓↓	—	↓↓	—	—	—
Ugt1a5	—	↑↑	↑↑	↑↑	—	—	—
Ugt1a6	—	—	↓↓	↓↓	↓↓	↓↓	—
Ugt1a9	—	—	—	↓↓	—	—	↓↓
Ugt2a3	—	—	—	↓↓	—	—	—
Ugt2b1	—	—	↓↓	↓↓	—	—	—
Ugt2b5	—	—	—	—	—	—	—
Ugt2b34	—	—	↓↓	↓↓	—	—	—
Ugt2b35	—	↓↓	↓↓	↓↓	↓↓	↓↓	—
Sults							
Sult1a1	↑↑	—	—	↑↑	↑↑	—	—
Sult2a1/2	↑↑	—	—	—	↑↑	↑↑	—
Sult1d1	—	—	↓↓	↓↓	—	—	—
Sult3a1	—	↑↑	↑↑	—	—	—	—
Gsts							
Gsta1	—	↓↓	↓↓	↓↓	—	—	—
Gsta4	—	—	↓↓	↓↓	—	—	—
Gstm2	↑↑	—	—	—	—	↑↑	—
Gstp1	↓↓	—	—	—	—	—	—
Signaling							
AhR	ND	ND	—	—	ND	ND	ND
CAR	ND	ND	↓↓	—	ND	ND	ND
PXR	ND	ND	—	↓↓	ND	ND	ND
PPARα	ND	ND	—	↓↓	ND	ND	ND
Nrf2	ND	ND	—	—	ND	ND	ND
ERα	ND	ND	↑↑	↑↑	ND	ND	ND
Pgc-1α	ND	ND	↓↓	—	ND	ND	ND
Cyp1a1	ND	ND	—	—	ND	ND	ND
Cyp2b10	ND	ND	↓↓	↓↓	ND	ND	ND
Cyp3a11	ND	ND	—	↓↓	ND	ND	ND
Cyp4a14	ND	ND	↓↓	↓↓	ND	ND	ND
Nqo1	ND	ND	—	—	ND	ND	ND

ND, not determined.

or 17 (Fig. 1A). Therefore, BPA glucuronidation was used as a marker reaction to characterize Ugt activity. On GD14, in vitro BPA-glucuronide formation was significantly decreased by 30% in livers of pregnant mice compared with virgin controls (Fig. 1C). There was no significant change in BPA-glucuronide formation between virgin and pregnant mice on GD17.

APAP is metabolized by UGT1A1, 1A6, 1A9, and 2B15 (Ugt35 in mice) in humans (Court et al., 2001; Mutlib et al., 2006). Downregulation of mRNA and/or protein expression for 1a6 and 1a9 was found in the present study. Therefore, we also tested whether APAP glucuronidation in vitro was altered in S9 fractions from livers of pregnant mice. However, no significant differences in APAP-glucuronide generation were observed between pregnant dams and virgin controls after 30- to 120-minute incubations (data not shown).

Hepatic Sult Expression and Function during Pregnancy and Lactation. Sults are cytosolic enzymes that are important in hormone regulation/metabolism and xenobiotic detoxification. Prior studies demonstrate marked gender differences in Sult mRNA expression (Alnouti and Klaassen, 2011). Because sex hormones are important in gender-divergent Sult levels, we hypothesized that pregnancy might alter their expression. Sult1a1, 2a1/2, and 3a1 mRNAs were increased 100 to 500% at various time points during pregnancy and lactation (Fig. 2A; Table 1). Consistently, upregulation of Sult1a1 protein expression was also found on GD14 and 17 (Fig. 2B). Conversely, the mRNA expression of Sult1d1 was decreased 40 to 60% in dams during late pregnancy. Messenger RNA expression of all Sults returned to control levels in dams after pups were weaning.

In addition to Ugts, Sult1a1 is also involved in the conjugation of APAP in mice. During pregnancy, mRNA and protein expression of Sult1a1 were upregulated in mice livers. Coordinately, these changes resulted in an increase of enzyme activity, proven by 20–40% higher APAP-sulfate formation in liver S9 fractions from pregnant mice on GD14 and 17 (Fig. 2C).

Hepatic Gst Expression during Pregnancy and Lactation. Gst enzymes conjugate chemotherapeutic drugs, insecticides, herbicides, and carcinogens with glutathione and also protect cellular macromolecules against electrophiles and products of oxidative stress (reviewed in Hayes et al., 2005). Similar to Ugts, Gsta1 and a4 mRNAs were reduced in livers of pregnant mice during mid to late gestation (Fig. 3A; Table 1). In particular, Gsta1 and a4 mRNAs were decreased by 60 to 80% between GD14 and 17. A similar trend for down-regulation of Gsta4 protein expression was also observed, although it was not statistically significant (Fig. 3B). Gstp1 mRNA also decreased in pregnant mice during early gestation (GD7). Conversely, Gstm2 mRNA was increased on GD7 and PND15.

Hepatic Transcription Factor Expression and Activity during Pregnancy and Lactation. Phase II enzymes are cross-regulated by AhR, CAR, PPARα, PXR, and Nrf2. Expression of each transcription factor was quantified on GD14 and 17. To assess changes in activity of each transcription factor, mRNA levels of prototypical target genes were also measured. Coinciding with maximal decreases in Ugts and Gsts, the mRNA expression of CAR, PXR, and PPARα were downregulated on either GD14 or 17. In contrast, the expression of AhR and Nrf2 mRNA was unchanged (Fig. 4A).
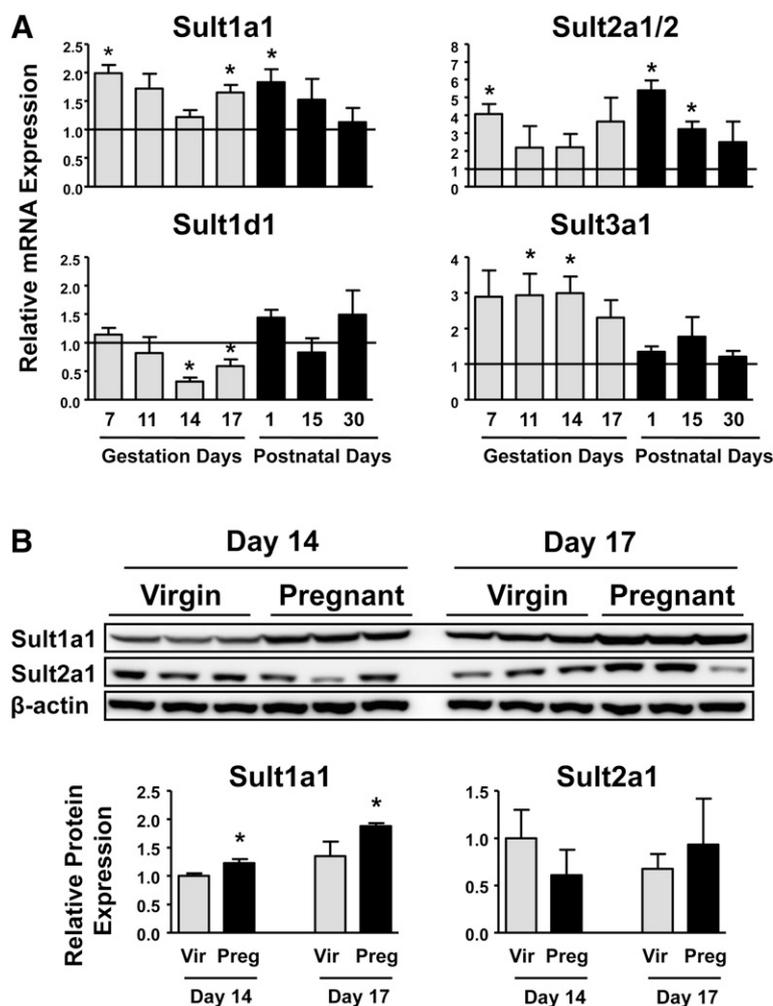
A

B

C

Fig. 2. Expression and activity of hepatic Sults in pregnant mice. (A) messenger RNA expression of Sult1a1, 2a1/2, 1d1, and 3a1 was quantified by the bDNA assay in time-matched pregnant and virgin mice at various gestational and postnatal days. Data were normalized to virgin mice (set to 1.0) and presented as mean ± S.E. (n = 3–4). (B) protein expression of Sult1a1 and 2a1 was quantified by Western blotting analysis in time-matched pregnant (GD14 and 17) and virgin mice (n = 3). (C) APAP (2 mM) was incubated with mouse liver S9 fractions isolated from time-matched pregnant (GD14 and 17) and virgin mice for 30 minutes in the presence of the cofactor PAPS (n = 5–6). *P < 0.05, statistically significant differences compared with time-matched virgin mice.

Consistently, mRNA and protein expression of Cyp2b10 (target gene of CAR) and Cyp4a14 (target gene of PPARα) were decreased by 60–90% compared with their controls (Fig. 4, A and B). Cyp3a11, which is a target gene of PXR, was also decreased by 40% at the mRNA level on GD17, although no difference in Cyp3a protein was observed (Fig. 4, A and B). Of note, the antibody used for quantification of Cyp3a11 was anti-Cyp3a4/3a1/3a11. Thus, additional Cyp3a isoforms may be masking a potential decline in Cyp3a11 protein. No changes in Cyp1a1 (target gene of AhR) nor NAD(P)H quinone oxidoreductase 1 (Nqo1, target gene of Nrf2) mRNA or protein were observed.

Hnf4α and Pgc-1α are important transcriptional regulators that can interact with several transcription factors, including PPARα and CAR, to regulate the expression of hepatic metabolic enzymes and transporters (Wortham et al., 2007; Jover et al., 2009; Lu et al., 2010). The mRNA level of Pgc-1α was dramatically decreased by 60% at GD14 (Fig. 5). Hnf4α mRNA expression was not significantly changed in the livers of pregnant mice at GD14/17 (data not shown).

The mRNA level of ERα was increased to 200–400% on GD14 and 17 (Fig. 5). The mRNA expression of ERβ and progesterone receptor was very low in mouse liver, and changes in expression were undetectable during pregnancy (data not shown).

Discussion

The present study investigated the regulation of phase II enzyme mRNA, protein, and function during pregnancy and lactation in mice. We have demonstrated downregulation of hepatic glucuronidation and upregulation of sulfation during pregnancy that corresponds with time-dependent changes in mRNA expression of metabolism genes. We have further shown parallel decreases in the expression and activity of the transcription factors CAR, PPARα, and PXR and their coactivator Pgc-1α. In contrast, the expression of the hepatic ERα receptor was enhanced in pregnant mice. Taken together, CAR, PPARα, PXR, and ERα are candidate transcriptional pathways for the regulation of phase II metabolism during pregnancy; however, the exact contribution of each nuclear receptor needs to be tested directly.

Microarray analysis of livers from pregnant rats near parturition has demonstrated decreases in expression of several phase I enzymes, phase II enzymes including Ugt1a6 and 1a7, Gst alpha and mu genes, and the nuclear receptor CAR (He et al., 2007). In a separate study, it was shown that protein expression and enzyme activities of several Ugt isoforms were reduced in maternal rat livers in the absence of changes in mRNA (Luquita et al., 2001). As a result, post-translational regulation was suggested as a mechanism
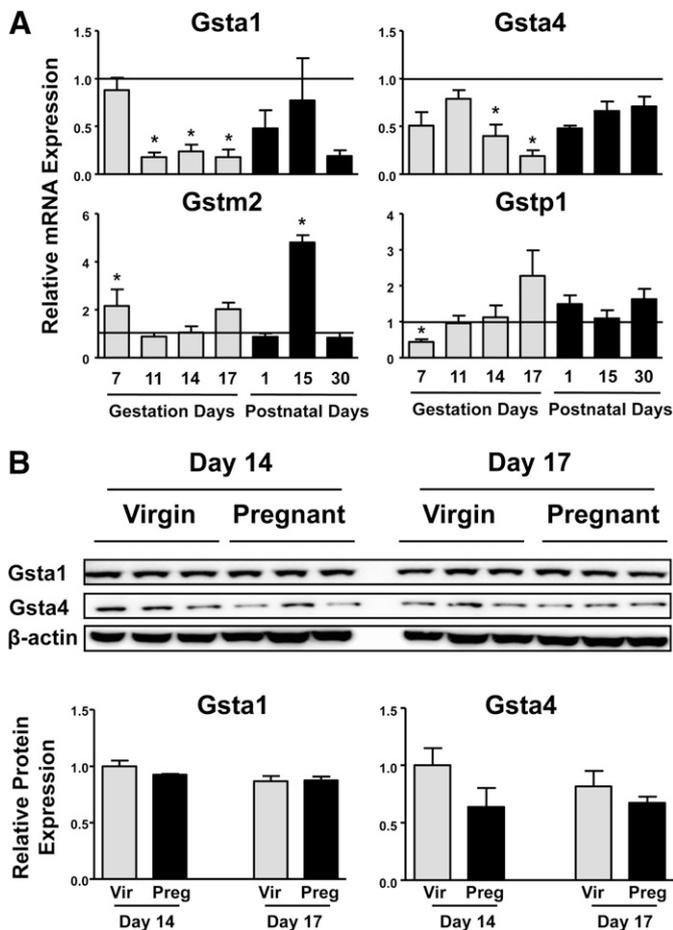
Fig. 3. Expression of hepatic Gsts in pregnant mice. (A) messenger RNA expression of Gsta1, a4, m2, and p1 was quantified by the bDNA assay in time-matched pregnant and virgin mice at various gestational and postnatal days. Data were normalized to virgin mice (set to 1.0) and presented as mean ± S.E. (n = 3–4). (B) protein expression of Gsta1 and a4 was quantified by Western blotting analysis in time-matched pregnant (GD14 and 17) and virgin mice (n = 3). *P < 0.05, statistically significant differences compared with time-matched virgin mice.

regulating phase II metabolism during pregnancy. We show coordinate decreases in Ugt and Gst mRNAs in a time-dependent manner. Therefore, we conclude that regulation of Ugt transcription in mice is likely to be the mechanism for protein and functional changes and that prior rat studies may reflect differences between species or experimental design and strengthen the use of a time course in this study.

The transcription factors CAR and PXR often share similar xenobiotic responsive elements leading to an overlapping set of downstream genes (Wang and LeCluyse, 2003; Tolson and Wang, 2010). For example, phase II enzymes such as Ugt1a1, 1a6, 1a9, 2b1, 2b5, and Gsta1/2 are regulated by CAR and PXR activation in rodents (Chen et al., 2003; Maglich et al., 2004; Hayes et al., 2005; Buckley and Klaassen, 2009a; Tolson and Wang, 2010; Aleksunes and Klaassen, 2012). Consistently, in this study, these isoforms were downregulated during pregnancy and corresponded with reduced CAR and PXR signaling.

PPARα is one of the key transcriptional pathways responsible for regulating hepatic lipid metabolism. Chemical activation of PPARα can induce some Ugts and Gsts (Alnouti and Klaassen, 2008; Knight et al., 2008). Similar to CAR and

PXR, there is a downregulation of PPARα mRNA in mouse liver during pregnancy, which may contribute to the regulation of phase II genes (Sweeney et al., 2006; Papacleovoulou et al., 2011). In addition to downregulating nuclear receptors, pregnancy also reduces the mRNA expression of Pgc-1α, which is a transcription coactivator of PPAR signaling and a key regulator of cellular energy metabolism (Sweeney et al., 2006; Jover et al., 2009; Fig. 5). Several previous studies have also pointed to HNF4α as a transcriptional regulator of metabolic enzymes and transporters in the liver (Wortham et al., 2007; Jover et al., 2009; Lu et al., 2010). However, we observed no change in Hnf4α mRNA in pregnancy. Further research is needed to definitively demonstrate the mechanistic role of each transcription factor in regulating phase II genes during pregnancy.

Pregnancy causes high levels of circulating sex hormones. Serum estradiol and progesterone were maximally increased between GD14 and 17 (data not shown). Elevated levels of hormones coupled with induction of ERα mRNA may be involved in the regulation of other nuclear receptors. A prior study demonstrated the downregulation of PXR and CAR in estradiol benzoate-treated rats (Choi et al., 2011). In addition, Cyp2b10 (CAR target gene) is induced by estradiol and repressed by progesterone in primary mouse hepatocytes, suggesting divergent regulation of this pathway by sex hormones (Kawamoto et al., 2000). Interestingly, CAR can inhibit ER-mediated signaling pathway by interfering with coactivators and direct interaction of ER and CAR in human hepatoma cells (Min et al., 2002). Therefore, increased estradiol and progesterone signaling may be one of possible mechanisms for modulating other nuclear receptor pathways during pregnancy.

The plasticizer BPA undergoes extensive glucuronidation in humans and is largely excreted in its conjugated form. In the present study, BPA glucuronidation was reduced in S9 fractions from livers of pregnant mice, which is likely due to downregulation of Ugt2b1 and Ugt2b35 expression. Similar functional changes in BPA conjugation have been reported in microsomes from pregnant rat livers (Matsumoto et al., 2002). In addition, BPA-glucuronide excretion into bile is reduced in pregnant rats due to downregulation of the multidrug resistance-associated protein 2 transporter (Inoue et al., 2005). It is likely that downregulation of BPA glucuronidation and transport may be the mechanisms governing reduced biliary excretion of BPA during pregnancy in rodents. In contrast to BPA, glucuronidation of APAP did not significantly change in S9 fractions from pregnant mouse livers. Clinically, the oral clearance of APAP has been reported to be 58% higher in the pregnant women compared with nonpregnant women of comparable age and attributed to increased Ugt activity (Miners et al., 1986). It is unknown whether this contradictory finding is the result of differential regulation between species or APAP concentrations selected. Nonetheless, additional studies are needed to test the effects of pregnancy on the phase II metabolism of specific chemicals and drugs.

The majority of Ugt isoforms are downregulated in livers of pregnant mice, with the exception of Ugt1a5. In pregnant mice, Ugt1a5 mRNA increased 50 to 100% during mid to late gestation (Fig. 1A). Ugt1a5 mRNA has been shown to be markedly induced in livers of hypophysectomized female mice treated with estradiol (Buckley and Klaassen, 2009b).
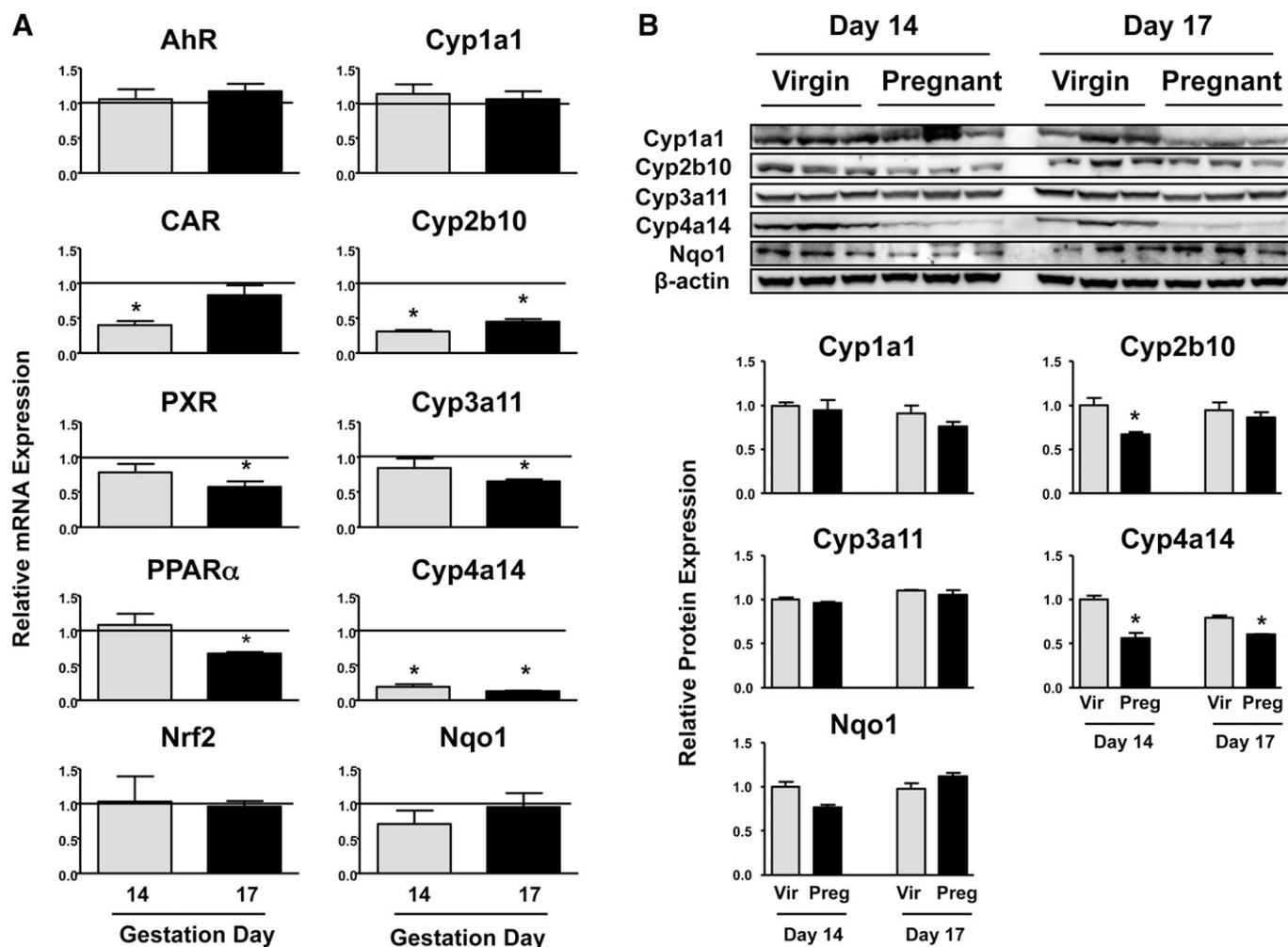
Fig. 4. Expression of hepatic transcriptional factors and prototypical target genes. (A) messenger RNA expression of AhR, CAR, PXR, PPARα, and Nrf2 and their target genes Cyp1a1, 2b10, 3a11, 4a14, and Nqo1 was quantified by the bDNA assay in time-matched pregnant (GD14 and 17) and virgin mice. Data were normalized to virgin mice (set to 1.0) and presented as mean ± S.E. (n = 3–4). (B) protein expression of Cyp1a1, 2b10, 3a11, 4a14, and Nqo1 was quantified by Western blotting analysis in time-matched pregnant (GD14 and 17) and virgin mice (n = 3). *P < 0.05, statistically significant differences compared with time-matched virgin mice.

Furthermore, it has been demonstrated that the human ortholog of Ugt1a5, UGT1A4, is upregulated by 17β-estradiol in human hepatoma cells via the estrogen receptor and specificity protein 1 transcription factor (Chen et al., 2009). Moreover, the upregulation of this UGT isoform is suggested to be responsible for enhanced clearance of the antiseizure drug lamotrigine during pregnancy (Tran et al., 2002; de Haan et al., 2004; Pennell et al., 2004). Because estradiol levels are elevated in pregnancy, direct stimulation of Ugt1a5 transcription by estradiol may explain the regulation of this gene in an opposing direction to other Ugts.

Increased Sult mRNA expression during pregnancy may be attributed to the stimulatory effects of sex hormones. For example, exogenous administration of 17β-estradiol to female mice that have undergone either hypophysectomy or gonadectomy induces hepatic expression of Sult2a1/2 and 3a1 mRNA (Alnouti and Klaassen, 2011). Likewise, the rat Sult1a1 promoter is transactivated in hepatocytes in vitro in response to treatment with the glucocorticoid dexamethasone (Duanmu et al., 2001). Glucocorticoid and androgen receptors also activate the same region of the Sult1a1

promoter in cultured hepatocytes (Fang et al., 2003). Because glucocorticoids, androgens, and progesterone share the same hormone response element (an inverted repeat separated by three nucleotides) (Ham et al., 1988; Beato, 1989; Fang et al., 2003), elevated progesterone concentrations may be involved in upregulation of Sult1a1 mRNA in pregnant mouse livers.

It is well known that the plasma concentrations of some drugs change during pregnancy. However, most of these clinical reports are related to cytochrome P450-mediated metabolism of drugs (Anderson, 2005). Information regarding pregnancy-induced pharmacokinetic changes of phase II-metabolized drugs is limited. Clinical pharmacokinetic changes in lamotrigine, zidovudine, morphine, and oxazepam disposition have been attributed to altered phase II metabolism during pregnancy (Anderson, 2005). UGT2B7 and 2B15 upregulation has been proposed as the mechanism responsible for the increased clearance of zidovudine, morphine, and oxazepam in pregnant women (Tomson et al., 1979; Gerdin et al., 1990; Watts et al., 1991; O'Sullivan et al., 1993). Likewise, the oral clearance of labetalol is increased in
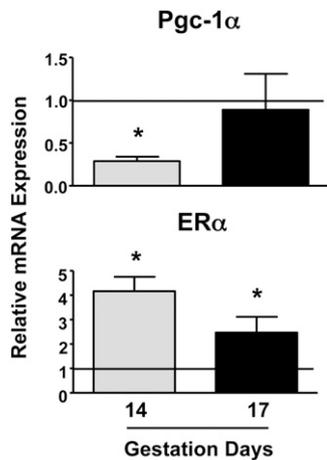
Pgc-1α

Fig. 5. Messenger RNA expression of hepatic Pgc-1α and ERα in pregnant mice. Messenger RNA expression of Pgc-1α and ERα was quantified by qPCR in time-matched pregnant (GD14 and 17) and virgin mice. Data were normalized to virgin mice (set to 1.0) and presented as mean ± S.E. (n = 3–4). *P < 0.05, statistically significant differences compared with time-matched virgin mice.

pregnant women (Rogers et al., 1990). Further investigation has suggested a potential role for progesterone in regulating labetalol elimination by modulating the hepatic expression and function of UGT1A1 during pregnancy (Jeong et al., 2008). Conversely, we observed downregulation of Ugt1a1 mRNA during pregnancy in the present study, suggesting that this isoform may be differentially regulated between species. Therefore, extrapolation of rodent data from developmental toxicity studies to humans should be done cautiously because of potential differences in maternal metabolism and clearance.

The hepatic mRNA expression of Ugts and Gsts and their upstream transcription factors CAR, PXR, and PPARα were downregulated in parallel in mice during pregnancy. In addition, enhanced levels of Ugt1a5 mRNA and various Sult isoforms as well as ERα were observed. However, to more definitively demonstrate the mechanistic link between altered expression of transcriptional factors and metabolic enzymes, additional experiments such as direct treatment of hepatocytes with sex hormones and gene knockout/knockdown studies are needed. Collectively, changes in phase II metabolism may alter chemical disposition and the likelihood of fetal drug exposure during pregnancy.

Authorship Contributions

Participated in research design: Wen, Aleksunes.
Conducted experiments: Wen, Donepudi.
Contributed new reagents or analytic tools: Slitt, Thomas, King.
Performed data analysis: Wen, Donepudi.
Wrote or contributed to the writing of the manuscript: Wen, Aleksunes.

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